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Differential induction of type I interferon by type I and type II feline coronaviruses in vitro

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ABSTRACT

Feline infectious peritonitis (FIP) is a feline coronavirus (FCoV)-induced fatal disease in wild and domestic cats. There are two FCoV serotypes. Both type I and II FCoV can replicate in *Felis catus* whole fetus (fcwf)-4 cells, but the replicability of type I FCoV in feline cell lines is lower than that of type II FCoV, the reason for which is unclear. Inhibition of IFNβ production by non-structural and structural proteins, excluding spike protein has been reported in many coronavirus infections. In this study, we investigated whether IFNβ is involved in the difference in replicability in feline cell lines between types I and II FCoV. When fcwf-4 cells were infected with FCoV, the virus titer of type II FCoV in the culture supernatant was higher than that of type I FIPV. When the IFNβ expression level in FCoV-infected fcwf-4 cells was semi-quantitatively analyzed, infection with type I FIPV, excluding type I FIPV UCD-1, highly induced IFNβ expression. In contrast, induction of IFNβ by type II FCoV infection was significantly lower than that by type I FIPV. In addition, when fcwf-4 cells were adsorbed by FIPV and then stimulated with Poly(I:C), type II FCoV infection inhibited Poly(I:C)-induced IFNβ gene expression. Also, the proliferation of type I FIPV was enhanced by a IFN inhibitor. These findings clarified that, unlike type I FIPV, type II FCoV strongly inhibits IFNβ expression in infected cells. It was also suggested that the IFNβ-inducing ability is different among type I FIPV strains.

1. Introduction

Coronaviruses are single-stranded positive-sense RNA viruses in the subfamily *Coronavirinae* of the family *Coronaviridae*. *Coronavirinae* has been classified into *alpha-*, *beta-*, *gamma-*, and *deltacoronavirus* (Su et al., 2016). Feline coronavirus (FCoV) belongs to *alphacoronavirus*, which includes porcine transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), and canine coronavirus (CCoV). FCoV has been classified into two biotypes: weakly pathogenic feline enteric coronavirus (FECV; avirulent FCoV) and strongly pathogenic feline infectious peritonitis virus (FIPV; virulent FCoV) (Pedersen NC, 2009). Cats that developed FIP were affected in several organs, including the liver, lungs, spleen, and central nervous system, forming lesions accompanied by necrosis and pyogenic granulomatous inflammation. In some cats, pleural effusion and ascitic fluid accumulated (Pedersen NC, 2009). The FCoV virion is mainly composed of nucleocapsid (N), envelope (E), membrane (M), and peplomer spike (S) proteins (Tekes and Thiel, 2016). FECV and FIPV are classified into 2 serotypes, type I and II FCoV, based on differences in the amino acid sequence of S protein (Motokawa et al., 1995).

Both type I and II FCoV can replicate in *Felis catus* whole fetus (fcwf)-4 cells, but the replicability of type I FCoV in feline cell lines is lower than that of type II FCoV, i.e., the amount of virus produced by type I FIPV-infected cells is approximately 1/1000 of that by type II FCoV-infected cells (Pedersen et al., 1984). Type I FCoV, except for some of types of I FIPV, cannot proliferate in cultured cells. The reason for the difference in replicability between the types is unclear.

Interferon (IFN) is a cytokine that induces an antiviral state in cells and influences via host cells (Coccia and Battistini, 2015). IFN is divided into type I, II, and III IFN. Type I IFN is an antiviral factor produced in response to viral infection. IFNα/β of type I IFN is produced by several cell types and plays an important role in the early defense mechanism against viral infection. For viruses to replicate in cells, inhibition of the antiviral action of IFNα/β (type I IFN response) is necessary. The mechanism of inhibition of type I IFN response by IFNβ has been closely investigated in several viruses. For example, in TGEV, PEDV, murine hepatitis virus, severe acute respiratory syndrome (SARS)-CoV, and middle east respiratory syndrome (MERS)-CoV, it has been reported that structural proteins, excluding non-structural proteins and spike protein, inhibit IFNβ production (Case et al., 2018, 2016; Ding et al., 2017; Hu et al., 2017; Li et al., 2016; Lu et al., 2011; Lui et al., 2016; Zhang et al., 2016). However, it is unclear whether type I IFN responses are inhibited by FCoV.

In this study, we examined whether IFNβ is involved in the difference in replicability between type I and II FCoV in a feline cell line.

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2. Materials and methods

2.1. Cell cultures and viruses

fcwf-4 cells (kindly supplied by Dr. M. C. Horzinek of the State University of Utrecht) were grown in Eagle's minimum essential medium containing 50% L-15 medium, 5% fetal calf serum (FCS), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The fcwf-4 cells were cultured for at least 70 passages. The fcwf-4 cells were preliminarily examined regarding i) susceptibility to type I FIPV and type II FCoV, ii) production of type I IFN, and iii) susceptibility to type I IFN. Type I FIPV KU-2 was isolated in our laboratory. Type I FIPV UCD-1, UCD-2, UCD-4, and Black were kindly provided by Dr. J. K. Yamamoto from the University of Florida. Type II FCoV 79–1146 was supplied by Dr. M. C. Horzinek of the State University Utrecht, the Netherlands. Type II FCoV 79–1683 was supplied by Dr. A. J. Mckeirnan of Washington State University. These viruses were grown in fcwf-4 cells at 37 °C

2.2. Compound

JAK1 inhibitor, Ruxolitinib, was used as inhibitors of type I IFN signaling. Ruxolitinib was purchased from Cayman chemical (U.S.A.). Ruxolitinib was prepared as 10 mM stocks in dimethyl sulfoxide (DMSO). On the day of the experiments, Ruxolitinib was diluted to the desired concentrations in medium.

2.3. Inoculation of fcwf-4 cells with FCoV

A monolayer culture of fcwf-4 cells in a plastic 24-well multi-plate was inoculated with FCoV at MOI≒0.01. The cells were adsorbed by the virus at 37 °C for one hour and washed 3 times with serum-free medium. Culture medium was added to the culture plate at 1 ml/well and the cells were cultured. The culture supernatant and cells were collected 24 h after viral adsorption (25 h after viral inoculation). The virus titer (TCID50) in the culture supernatant was determined by the method of Reed and Muench (Ramakrishnan, 2016) with fcwf-4 cells. Cells were used to measure the gene expression levels of the FCoV N gene and feline IFNβ gene.

2.4. Preparation of UV-inactivated virus

To inactivate viral replicability, FCoV (1×10^5 TCID50/ml) was irradiated with UV at an intensity of 18 J/cm^2. It was confirmed that no CPE occurred in fcwf-4 cells inoculated with the UV-irradiated FCoV. Viral inoculation and collection of cells were performed as described above.

2.5. Inoculation of fcwf-4 cells with FCoV and stimulation of the cells with polyinosinic-polycytidylic acid (Poly(I:C))

A monolayer culture of fcwf-4 cells at 1×10^5 cells/well in a plastic 24-well multi-plate was inoculated with and adsorbed by FCoV at MOI≒0.1 at 37 °C for 1 h. After adsorption, the virus suspension was removed and the cells were washed 3 times with serum-free medium. After washing, culture medium was added at 1 ml/well followed by addition of 50 μl/well of Poly(I:C) (1 μg/ml, Sigma-Aldrich, U.S.A.) in a complex with Lipofectamine 2000 (Thermo Fisher Scientific K.K., U.S.A.). The cells were collected 12 h after viral adsorption (13 h after viral inoculation). Cells were used to measure the gene expression levels of the FCoV N gene and feline IFNβ gene.

2.6. Cytotoxic effects of Ruxolitinib

The fcwf-4 cells were seeded on 96-well plates. Ruxolitinib was added in triplicate to the wells. The cells were incubated at 37 °C for 48 h. After incubation, the culture supernatants were removed, WST-8 solution (Kishida Chemical, Japan) was added, and the cells were returned to the incubator for 1 h. The absorbance of formazan produced was measured at 450 nm with a 96-well spectrophotometric plate reader, as described by the manufacturer. Percentage viability was calculated using the following formula: Cytotoxicity (%) = [(OD of Ruxolitinib-untreated cells - Ruxolitinib-treated cells) / (OD of Ruxolitinib-untreated cells)] × 100.

2.7. IFN signaling inhibition assay

Confluent fcwf-4 cell monolayers were cultured in medium containing Ruxolitinib at the indicated concentrations in 24-well multi-plates at 37 °C for 2 h. Cell supernatant were supplemented with 10^4 units/well of recombinant feline interferon ω (Intercat®, Toray industries, INC., Japan). The cells were incubated at 37 °C for 48 h. After incubation, cells were collected and used to measure the gene expression levels of the feline Mx gene.

2.8. Ruxolitinib treatment and virus inoculation

Confluent fcwf-4 cell monolayers were cultured in medium containing Ruxolitinib at the indicated concentrations in 24-well multi-plates at 37 °C for 2 h. The cells were inoculated with FCoV at MOI ≒ 0.01 in the presense of Ruxolitinib. After virus adsorption for one hour, the cells were washed 3 times with serum-free medium. Culture medium containing Ruxolitinib was added to the culture plate at 1 ml/well and the cells were cultured at 37 °C. The culture supernatant were collected 24 h after viral adsorption. The virus titer in the culture supernatant was determined.

2.9. RNA isolation and cDNA preparation

Total cellular RNA was extracted from fcwf-4 cells and feline monocytes using a High Pure RNA Isolation Kit (Roche Diagnostics, Switzerland) according to the manufacturer's instructions. RNA was dissolved in elution buffer. Using the total cellular RNA as a template, cDNA was synthesized using the PrimeScript™ 1st strand cDNA Synthesis Kit (Takara Bio Inc., Japan). Reverse transcription was performed in a 20-μl final volume containing 1 μl of oligo(dT) primer (50 μM). The resulting solution was incubated at 42 °C for 30 min to synthesize cDNA.

2.10. Measurement of feline GAPDH, FCoV N gene, feline IFNβ gene and Mx gene expression

cDNA was amplified by PCR using specific primers for feline GAPDH, FCoV N gene and feline IFNβ gene. The primer sequences are shown in Table 1. PCR was performed in a total volume of 50 μl. One microliter of sample cDNA was mixed with 25 μl of Quick Taq HS DyeMix (TOYOBO Co., Japan), 2 μl of 10 μM primer mix, and 22 μl of distilled water. Using a PCR Thermal Cycler Dice (Takara Bio Inc., Japan), the DNA was amplified at 94 °C for 20 s, followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 45 s, and synthesis at 72 °C for 45 s, with a final extension at 72 °C for 5 min. The PCR products were resolved by electrophoresis on 3% agarose gels. The gels were incubated with ethidium bromide solution (1 μg/ml), and bands were visualized using a UV transilluminator at 312 nm and photographed. The band density was quantified under appropriate UV exposure by video densitometry using Image J software (NIH, USA). The FCoV N gene, feline IFNβ gene and feline Mx gene were semi-quantitatively analyzed in terms of the relative density value to that for the housekeeping gene GAPDH.

Table 1
Sequences of PCR primers for feline GAPDH, IFNβ, and FCoV N.

	Orientation	Nucleotide sequence	Length	Reference
GAPDH	Forward	5′-AATTCCACGGCACAGTCAAGG-3′	97	Avery and Hoover (2004)
	Reverse	5′-CATTTGATGTTGGCGGGATC-3′		
IFNβ	Forward	5′-TGGAATGAGACCACTGTTGAGAA-3′	69	Robert-Tissot et al. (2011)
	Reverse	5′-GGATCGTTTCCAGGTGTTCCT-3′		
Mx	Forward	5′-ACCAGAGCTCGGGCAAGAG-3′	96	Robert-Tissot et al. (2011)
	Reverse	5′-TTCAGCACCAGAGGACACCTT-3′		
FCoV N	Forward	5′-ATTGATGGAGTCTTCTGGGTTG-3′	362	GenBank Accession No. X56496
	Reverse	5′-TTGGCATTCTTAGGTGTTGTGTC-3′		

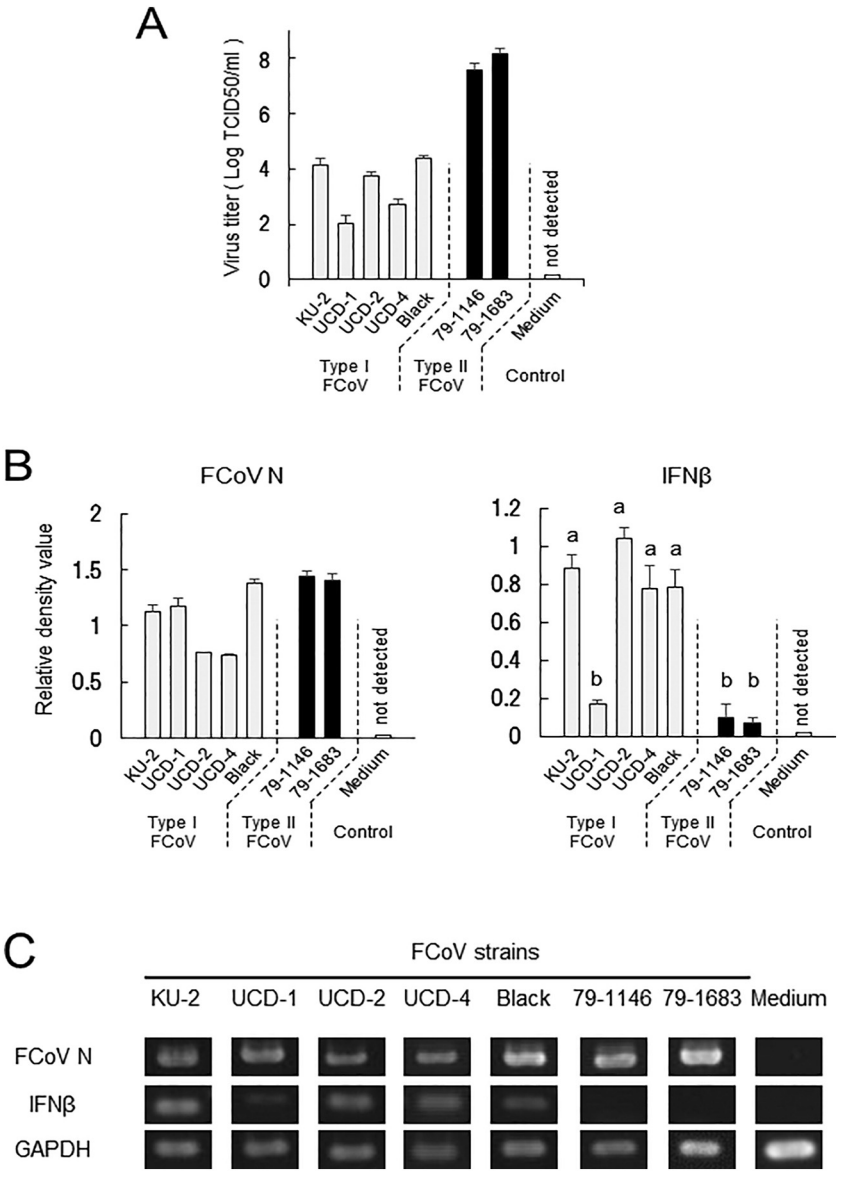

Fig. 1. Type I IFN response in FCoV-infected cells. A monolayer culture of fcwf-4 cells in a plastic 24-well multi-plate was inoculated with and adsorbed by FCoV at MOI≒0.01 at 37 °C for 1 h. The culture supernatant and cells were collected 24 h after viral adsorption (25 h after inoculation). (A) Virus titers in the culture supernatants. (B and C) FCoV N and IFNβ gene expression levels in fcwf-4 cells. The gene expression levels were semi-quantitatively analyzed as a relative density value to the GAPDH gene expression level. The results are the mean ± SE. Data represent three independent experiments. Different alphabets are significantly different at $p < .05$.

2.11. Statistical analysis

Data from multiple groups were analyzed with Bartlett's test followed by Tukey's test.

3. Results

3.1. Type I IFN response in FCoV-infected cells

Replicability in fcwf-4 cells was compared between type I and II FCoV. At 24 h after viral adsorption, virus production of all FCoV strains was detected in the culture supernatant, and the FCoV N gene was expressed in infected cells (Fig. 1). No significant difference was noted in the FCoV N gene expression level in the infected cells among the viral strains, but the virus titer of type II FCoV in the culture supernatant was higher than that of type I FIPV. Induction of the IFNβ gene by FCoV infection was evaluated (Fig. 1B and C). The IFNβ expression level significantly increased in type I FIPV-infected cells, excluding cells infected with type I FIPV UCD-1, compared with in type II FCoV-infected cells.

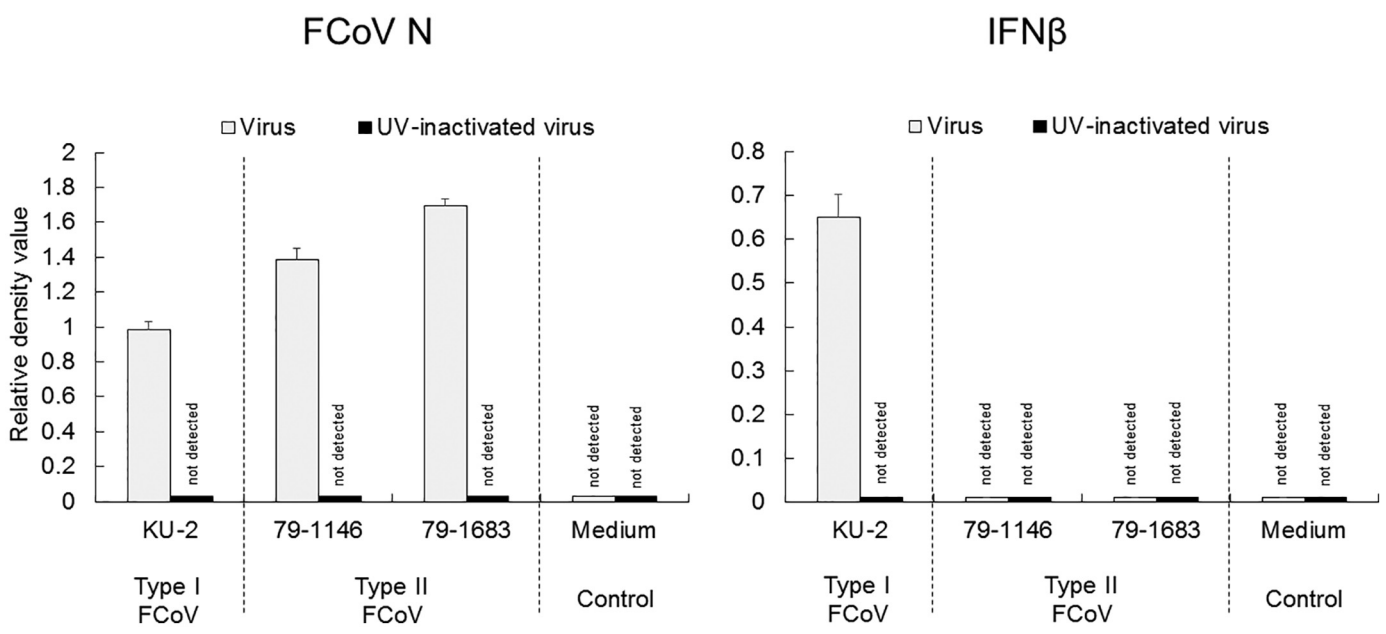

Fig. 2. IFNβ-inducing ability of UV-inactivated virus. Fcwf-4 cells were infected with virus (gray bar) or UV-inactivated virus (black bar). After a 24-h incubation, cells were collected 24 h after viral adsorption (25 h after inoculation). The results are the mean ± SE. Data represent three independent experiments.

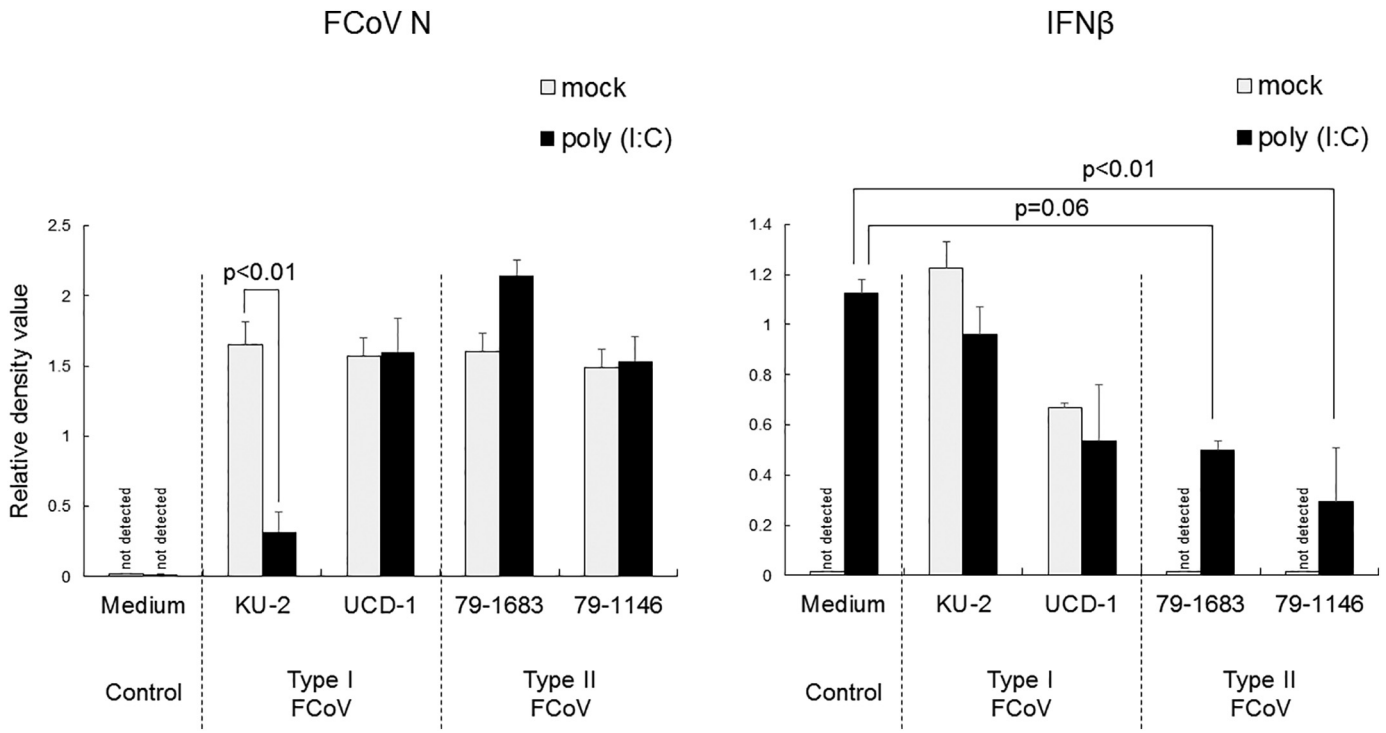

Fig. 3. Influence of FCoV infection on Poly(I:C)-induced IFNβ gene expression in fcwf-4 cells. After adsorption by FCoV, cells were cultured in medium containing Poly(I:C) (1 μg/ml) (gray bar) or not containing Poly(I:C) (black bar). The cells were collected 12 h after viral adsorption (13 h after inoculation) and RNA was extracted. The FCoV N gene and IFNβ gene expression levels were measured using the RT-PCR method. The gene expression levels were semi-quantitatively analyzed as a relative density value to the expression level of the house-keeping gene GAPDH. The results are the mean ± SE. Data represent three independent experiments.

The necessity of viral replication for IFNβ gene expression in FCoV-infected cells was investigated. In cells inoculated with non-inactivated type I FIPV KU-2, the FCoV N gene and the IFNβ gene were expressed. In cells inoculated with UV-inactivated type I FIPV KU-2, which lost replicability, no expression of the FCoV N gene or IFNβ gene was noted (Fig. 2). In cells inoculated with non-inactivated type II FCoV, the FCoV N gene was expressed but the IFNβ gene was not. In cells inoculated with UV-inactivated type II FCoV, no expression of the FCoV N gene or IFNβ gene was noted, similar with in cells inoculated with UV-inactivated type I FIPV KU-2.

3.2. Influence of FCoV infection on the IFNβ gene induced by Poly(I:C)

Inhibition of Poly(I:C)-induced IFNβ gene expression by FCoV was examined. After viral adsorption, the fcwf-4 cells were stimulated with Poly(I:C). The FCoV N gene expression level was not changed by

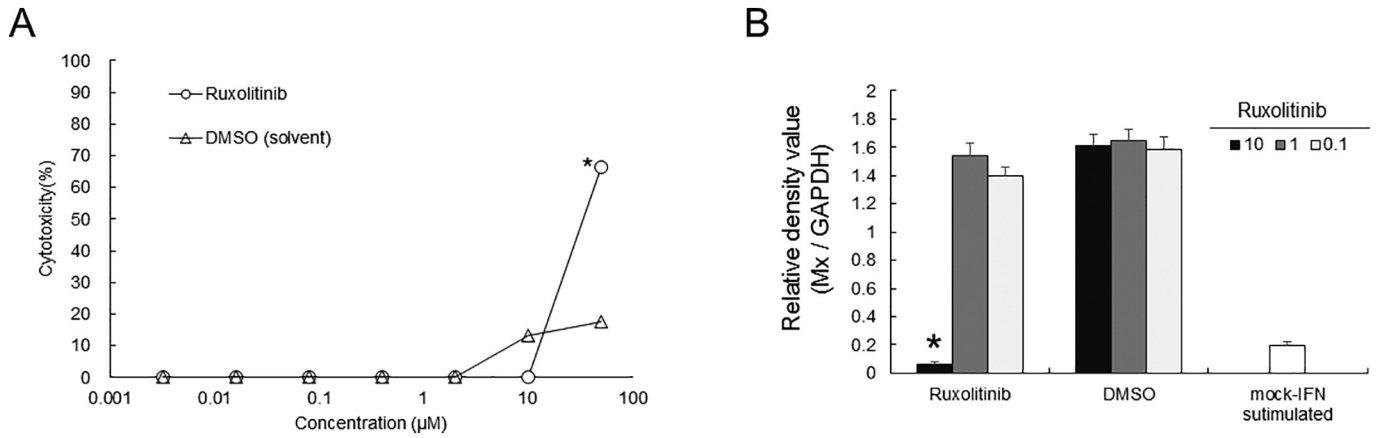

Fig. 4. Verification of Ruxolitinib cytotoxicity and ability to IFN signalin effects of Ruxolitinib in fcwf-4 cells. Data represent three independent experiments. (B) Mx gene expression levels in fcwf-4 cells. The gene expression levels were semi-quantitatively analyzed as a relative density value to the GAPDH gene expression level. The results are the mean ± SE. Data represent three independent experiments. *: $p < .05$ v.s. DMSO.

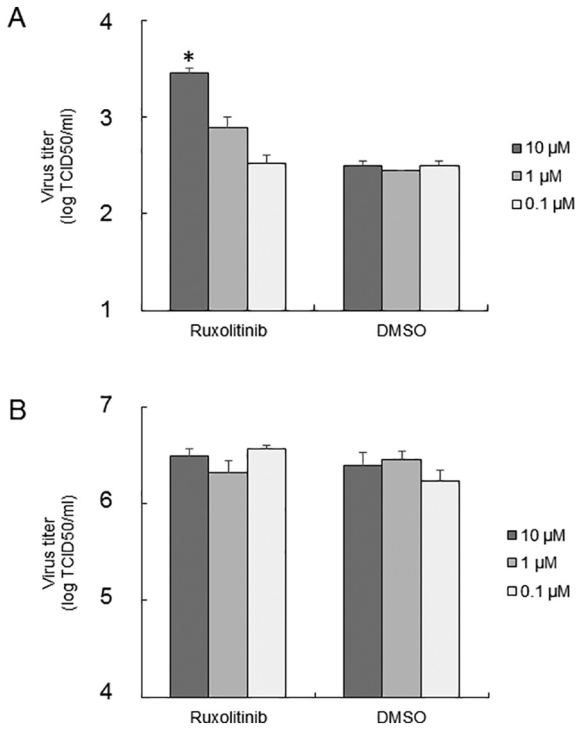

Fig. 5. Effects of Ruxolitinib on FCoV replication. The fcwf-4 cells were cultured in medium containing Ruxolitinib at 37 °C for 2 h. The cells were inoculated with FCoV in the presense of Ruxolitinib. After virus adsorption, culture medium containing Ruxolitinib was added to the well. (A) Virus titers of type I FCoV KU-2 in the culture supernatants. (B) Virus titers of type II FCoV 79–1146 in the culture supernatants. The results are the mean ± SE. Data represent three independent experiments. *: $p < .05$ v.s. DMSO.

stimulation with Poly(I:C) in cells infected with type I FIPV UCD-1, type II FCoV 79-1146, or type II FCoV 79-1683 (Fig. 3). In contrast, FCoV N gene expression was significantly reduced by stimulation with Poly(I:C) in type I FIPV KU-2 infected cells. When fcwf-4 cells without viral inoculation were stimulated with Poly(I:C), the IFNβ gene was induced. The IFNβ gene expression level significantly decreased in Poly(I:C)-stimulated FCoV-infected cells compared with that in cells without inoculation with type II FCoV 79-1146, and a similar tendency was noted for type II FCoV 79-1683 ($P = .06$). The IFNβ gene was expressed in cells infected with type I FIPV KU-2 or UCD-1, and no significant change in the IFNβ gene expression level was observed regardless of Poly(I:C) stimulation.

3.3. Effects of Ruxolitinib on FCoV replication

Ruxolitinib, a JAK 1 inhibitor, was reported to decrease the IFN susceptibility of cells and enhance viral proliferation. To examine whether IFN response influences the proliferation of type I FIPV after FCoV infection, ruxolitinib-treated cells were infected with type I FIPV.

The toxicity of ruxolitinib was examined in fcwf-4 cells. Ruxolitinib (50 μM) caused a significantly higher rate of cell damage than DMSO (solvent control, 0.5% DMSO) at the same dilution rate (Fig. 4A), and exhibited no significant cytotoxic effects at 10 μM.

To investigate whether ruxolitinib suppresses type I IFN signaling in fcwf-4 cells, fcwf-4 cells were stimulated with feline IFNω to examine the expression levels of the feline Mx gene. The expression level of Mx gene was higher in the IFNω-stimulated cells treated with DMSO as a solvent control than in the unstimulated cells (Fig. 4B). On the other hand, the expression level of Mx gene was significantly lower in 10 μM ruxolitinib-treated cells than in the solvent control cells. Specifically, type I IFN signaling was suppressed.

We next evaluated whether ruxolitinib influences the proliferation of FCoV. The virus titer in the culture supernatant of the type I FIPV KU-2-infected cells treated with ruxolitinib demonstrated a concentration-dependent increase, as compared with the solvent control (Fig. 5A). However, the virus titer in the culture supernatant of the type II FCoV 79–1146-infected cells exhibited no significant change in the presence of ruxolitinib at any concentration, as compared with the solvent control (Fig. 5B).

4. Discussion

Type I IFN is induced upon recognition of virus-derived nucleic acids by host cells through the pattern recognition receptor (PRR) (Kawai and Akira, 2006; Raftery and Stevenson, 2017). Secreted type I IFN induces IFN-stimulated genes through the IFN receptor on cells and JAK/STAT signaling pathway. Many viruses have different mechanisms to inhibit this type I IFN response, but whether FCoV inhibits induction of type I IFN is unknown. In this study, we investigated whether FCoV inhibits IFNβ production in a feline cell line.

Excluding type I FIPV UCD-1, type I FIPV infection highly induced IFNβ expression. The IFNβ gene expression level induced by type II FCoV infection was significantly lower than that induced by type I FIPV infection. In addition, Poly(I:C)-induced IFNβ gene expression was inhibited in cells infected with type II FCoV. These findings suggested that IFNβ induction is strongly inhibited in type II FCoV infection compared

with that in type I FIPV infection. Inhibition of type I IFN response by viruses is advantageous for their replication. IFN inhibitors promote viral replication in cells (Stewart et al., 2014). Similarly, the proliferation of type I FIPV was enhanced by a JAK1 inhibitor. These observations suggest that the proliferation of type I FIPV is influenced by the type I IFN response.

Type II FCoV was generated by genomic recombination between type I FCoV and type II CCoV (Decaro et al., 2009; Herrewegh et al., 1998; Terada et al., 2014). ORF1b, ORF2, and ORF3 of type II FCoV 79-1146, ORF1b, ORF2, ORF3, and ORF4 of type II FCoV 79-1683 are derived from type II CCoV, and all other genes are derived from type I FCoV. Thus, type II CCoV-derived genes recombined into type II FCoV may be involved in strong inhibition of IFNβ production by type II FCoV compared with that by type I FIPV. Of the genes in the recombined region, nsp14 (exonuclease), nsp15 (endoribonuclease), and nsp16 (2′-0-methyltransferase) present in the ORF1b of other coronaviruses have been reported to inhibit IFNβ gene expression (Case et al., 2016; Coccia and Battistini, 2015; Deng et al., 2017; Menachery et al., 2014; Zhang et al., 2016), and these nsps are considered to prevent recognition of viral RNA by host PRR. On the other hand, nsp14 of TGEV has been reported to be an IFNβ inducer (Zhou et al., 2017). These findings and our results suggest that nsp14, 15, and 16 also suppress IFNβ production by type I FIPVs. To clarify the mechanism of the difference in replicability between type I and II FCoV, it is necessary to closely investigate their functions.

Of type I FIPV, the IFNβ-inducing ability of FIPV UCD-1 was as low as that of type II FCoV, suggesting that the strength of IFN response induced by type I FIPV infection differs among strains. Difference in IFN-inducing ability within the same genotype of porcine reproductive and respiratory syndrome virus has been reported (Lee et al., 2004). The IFNβ-inducing ability was low only in FIPV UCD-1 among the type I FIPV strains, and this may have been due to viral replication. Indeed, the virus titer in the culture supernatant of type I FIPV UCD-1-infected cells was lower than that in the culture supernatants of cells infected with the other type I FIPV strains. In addition, in an experiment in which cells were inoculated with type I FIPV UCD-1 at a high infectivity titer, the IFNβ gene was induced to a level similar to that induced by type I FIPV KU-2. However, type I FIPV UCD-4 with a replicability level similar to that of type I FIPV UCD-1 strongly induced IFNβ expression. Further studies are required to clarify the relationship between the replicability of type I FIPV and IFNβ gene-inducing ability.

In conclusion, unlike type I FIPV, type II FCoV was found to strongly inhibit IFNβ expression in infected cells, and nsp14, nsp15, and nsp16 encoded in ORF1 may be involved in this difference between type I and II FCoV. It was also suggested that IFNβ-inducing ability differs among type I FIPV strains. Close investigation of the function of these nsps may reveal the mechanism behind the difference in replicability between type I and II FCoV, and resistance of FCoV to host natural immunity. In addition, whether similar results can be obtained in cats infected with FIPV should be examined. Furthermore, a method of culturing type I FECV in a cell line should be developed to investigate IFNβ production by type I FECV.

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